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CONTRACT NO: DAMD17-93-C-3116

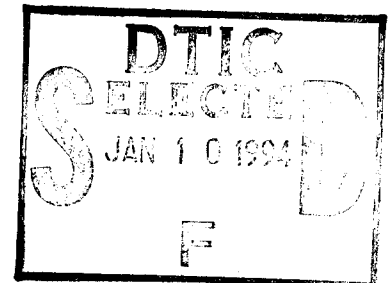
TITLE: MECHANISMS OF HYPERTENSION AFTER CROSS-LINKED
HEMOGLOBIN BLOOD-SUBSTITUTE TRANSFUSION

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REPORT DATE: December 1, 1994

TYPE OF REPORT: Midterm Report



PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 1 Dec 94		3. REPORT TYPE AND DATES COVERED Midterm Report
4. TITLE AND SUBTITLE Mechanisms of Hypertension After Cross-linked Hemoglobin Blood Substitute Transfusion			5. FUNDING NUMBERS DAMD17-93-C-3116	
6. AUTHOR(S) Michael J. Joyner, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mayo Foundation 200 First Street Southwest Rochester, Minnesota 55905			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES N/A				
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The USAMRDC has developed a "blood substitute" containing cross-linked human hemoglobin (XL-Hgb) in a physiologic saline solution. In animal models, this material can sustain life in the absence of red blood cells and is effective in resuscitating experimental animals from hypovolemic hemorrhagic shock. A persistent side-effect of XL-Hgb administration in animals has been marked arterial and pulmonary hypertension. The mechanism of this hypertension is unknown, but it is hypothesized that the XL-Hgb scavenges the endogenous vasodilating substance nitric oxide (NO). In experiments to date, our group has demonstrated that XL-Hgb administration 1) appears to blunt NO-mediated vasodilation in isolated blood vessels; 2) disrupts the normal metabolism of catecholamines from the adrenal medulla and sympathetic nerve endings; 3) blunts NO-mediated vasodilation <i>in vivo</i> ; 4) causes acute volume expansion and hypertension without the normally observed diuresis and natriuresis <i>in vivo</i> . Studies to date generally confirm the hypothesis that XL-Hgb interferes with NO function in isolated tissues and whole animals. Additionally, other poorly understood physiologic mechanisms may also contribute to the hypertension. Studies planned in the second half of this contract will continue to explore NO-mediated (and other) mechanisms which underlie the hypertension seen after XL-Hgb transfusion.				
14. SUBJECT TERMS Blood substitutes, hypertension, side effects, nitric oxide			15. NUMBER OF PAGES 19	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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Yes In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

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PI - Signature

Date

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INTRODUCTION

Nature of the Problem

A major threat to injured combat soldiers and civilian trauma victims is death due to exsanguination (14). In these patients, field replacement of blood loss with an oxygen-transporting volume-expanding solution (i.e., "blood substitute") could be life-saving (6,8,14,15,16). Additionally, such a blood substitute could solve a variety of logistical and other problems related to the collection, storage, and administration of red blood cells. Such solutions could also address concerns related to infectious disease transmission, and also to concerns related to a "shortage" of volunteer blood donors in the civilian world. In this context, a number of promising blood substitutes containing modified hemoglobin compounds are now being developed. The United States Army has developed a cross-linked hemoglobin (XL-Hgb) solution that can sustain life in animal models after massive blood loss or exchange transfusions (4,6,7,8,13,15,16). If safe and effective in humans, such a compound could be life-saving for injured soldiers. Additionally, this compound also represents an important "dual use" technology that could have vast implications in civilian medicine (5). However, one consistent observation made in animal models is that transfusion of XL-Hgb solutions causes marked systemic and pulmonary hypertension (6,8). The current concept is that XL-Hgb scavenges the endogenous vasodilator nitric oxide (NO), thereby limiting its ability to relax blood vessels (1,6,8,11,12,15,16). This action of XL-Hgb then causes marked systemic and pulmonary vasoconstriction and the resultant hypertension. In summary, the following key points should be remembered when evaluating the current state of hemoglobin-based blood substitutes:

- 1) Injured soldiers and civilian trauma victims could benefit from an oxygen-carrying volume-expanding solution administered in the field.
- 2) There are a host of logistical problems with blood transfusions in both military and civilian medicine.
- 3) Standard blood transfusions are frequently impractical in the field.
- 4) Currently available hemoglobin-based blood substitutes typically cause marked systemic and pulmonary vasoconstriction.
- 5) This vasoconstriction is thought to be due to interference with NO function.
- 6) The overall purpose of the studies conducted under the auspices of this contract (USAMRDC 91283001) has been to identify the mechanisms behind this hypertension so that possible counter-measures can be proposed.

Previous Work

There have been attempts to develop "blood substitutes" since the last century. The term "blood substitutes" is something of a misnomer, since blood performs a host of physiologic functions in addition to the transport of oxygen and maintenance of intravascular blood volume (15,16). In this context, a more appropriate name for "blood substitutes" would be "oxygen-carrying volume-expanding solution." However, the term "blood substitute" is the widely accepted parlance. Beginning in the 1930s there were attempts to make hemoglobin-containing blood substitutes (2,3). Since that time, a variety of solutions

have been developed and tested in animal models. Along these lines, there have been numerous demonstrations that animals could survive for up to several days in the absence of native red blood cells in the presence of hemoglobin-containing blood substitutes. Additionally, there have also been a variety of demonstrations suggesting that these compounds would be effective in the fluid resuscitation of individuals suffering from hypovolemic hemorrhagic shock. There have been several persistent problems shown with all hemoglobin-based blood substitutes: 1) limited intravascular half-life (7); 2) hypertension (3,6,8); 3) potential for long-term disruption of renal function; 4) development of a "pure" hemoglobin solution free of red blood cell stromal debris (4,13). Over the years, issues 1 and 2 have been addressed using several techniques. The intravascular half-life of these compounds has been prolonged by chemical cross-linking so that the hemoglobin (once liberated from the red blood cells) remains in a tetrameric unit and does not break down into dimers and monomers (4,13). The potential for renal damage is also reduced via the cross-linking process. Additionally, better purification of the hemoglobin and removal of red cell stromal debris from these products has markedly reduced potential renal complication (issues 2 and 4). In this context, one of the most important of the newer generation hemoglobin solutions currently under development by a variety of governmental and industrial concerns is the U.S. Army Research and Development Command's alpha-alpha 9 XL-Hgb (4,6,7,8,13). Several important features of this molecule deserve attention:

- 1) It currently represents the gold standard for purified hemoglobin.
- 2) Development of this product has contributed to the emerging industrial technologies required to produce large "batches" of purified hemoglobin for hemoglobin-based solutions.
- 3) The cross-linking technique employed results in a stable hemoglobin tetramer with only very small fractions of "other" hemoglobin-related compounds in the final solution.
- 4) Non-infectious material can be prepared from red blood cells infected with various viral contaminants including HIV.
- 5) A variety of transfusion protocols in animal models suggests that this molecule is effective in restoring blood volume in animals subjected to experimental hypovolemic hemorrhagic shock. Additionally, animals can survive for prolonged periods of time after complete exchange transfusions with cross-linked hemoglobin solutions.
- 6) The major continuing "toxicity" of cross-linked hemoglobin solutions given to animals appears to be systemic and pulmonary hypertension as a result of vasoconstriction.

Purpose of the Present Work

With this information as a background, the purpose of the work outlined in this contract is to better understand the mechanisms responsible for the hypertensive effects of XL-Hgb when given to animals. Four general areas of research have been proposed, and each of these is well underway to completion; project 1 (Dr. Katušić) uses classic pharmacologic techniques on isolated blood vessels to "pharmacodissect" the interactions of XL-Hgb and the nitric oxide pathway that regulate vascular tone in a variety of blood vessels. In project 2 (Drs. Rorie and Tyce) the basic concept under study is that nitric oxide is emerging as a key regulator and controller of catecholamine release from both the adrenal medulla and sympathetic nerves. During "shock trauma" situations there can be massive release of catecholamines

from the adrenal medulla and the sympathetic nerves. In this context, studies proposed in component 2 of this contract evaluate the interaction of XL-Hgb, nitric oxide, and catecholamine release from the adrenal medulla and sympathetic nerves. In project 3 (Dr. Joyner) studies are being conducted in an anesthetized canine preparation. Several lines of investigation on the interactions of XL-Hgb and blood pressure regulating systems are under scrutiny. In a large series of experiments (just recently conducted), the effects of XL-Hgb on baseline vascular tone in a variety of organ systems and on the vasodilator responses to a variety of pharmacologic compounds have been studied. The overall purpose of these studies is to determine if XL-Hgb has similar effects on all blood vessels *in vivo* to the same extent as those seen in project 1 (Dr. Katušić). Additionally, in upcoming studies the effects of XL-Hgb and baroreceptor regulation of blood pressure will also be evaluated. This is important because it has recently been suggested that NO plays a key role at various sites of the baroreflex arc. In project 4 (Dr. Romero) the effects of XL-Hgb on renal blood pressure regulating mechanisms are being evaluated. The concept is that volume expansion and hypertension normally evoke marked diuresis and natriuresis that requires intact NO pathways in the kidney. When these pathways are inhibited, sodium retention, volume expansion, and hypertension occur and normal renal blood pressure regulating mechanisms are disrupted. Thus far XL-Hgb, when given as a volume expander, is one of the only solutions that causes both volume expansion and hypertension without evoking natriuresis and diuresis. This is consistent with the concept that NO is a key mediator of these responses.

Methods and Approach

Overview

Four laboratories are collaborating on interrelated projects to address the mechanisms of hypertension after XL-Hgb blood substitute transfusion. These include:

- 1) *Dr. Katušić.* In Dr. Katušić's laboratory, standard *in vitro* pharmacologic techniques in isolated blood vessels are used to explore the pharmacologic effects of XL-Hgb on the normal contracting and relaxing factors which regulate blood vessel tone.
- 2) In Project 2 *Drs. Rorie and Tyce* are investigating the interactions of XL-Hgb, nitric oxide, and catecholamine release from the adrenal medulla and sympathetic nerves. They have a fully equipped laboratory capable of making these measurements. Many of the basic techniques to study catecholamine metabolism were developed in this lab.
- 3) In Project 3 *Dr. Joyner* is investigating the effects of XL-Hgb administration on *in vivo* pharmacologic responses related to the NO pathway and also on baroreflex regulation and systemic vascular resistance in anesthetized animals.
- 4) In Project 4 *Dr. Romero* is investigating the effects of volume expansion with XL-Hgb on renal blood pressure mechanisms. In these studies the relationship between renal perfusion pressure, renal blood flow, renal sodium excretion and renal volume excretion, will be explored in detail. Many of these techniques were developed and perfected in Dr. Romero's lab.

Technical Objectives

Objective 1. To determine if XL-Hgb transfusion inhibits the production and function of the endothelium-derived vasodilator NO.

Objective 2. To determine if the local vasoconstricting effects of XL-Hgb solutions are reinforced by increased release of catecholamines and vasoconstricting factors from the adrenal gland and postganglionic sympathetic nerves.

Objective 3. To determine if XL-Hgb disturbs local endothelial and sympathetic control of vascular tone in vivo and if baroreflex control of peripheral vascular resistance is altered after XL-Hgb.

Objective 4. To determine if hypertension after XL-Hgb transfusion is maintained in spite of "normal" renal hemodynamics due to a disruption of the NO-mediated pressure-induced natriuresis and diuresis usually seen during volume expansion and hypertension.

Techniques

Animal Model. Studies have been conducted in anesthetized 8-12 kg dogs and isolated canine blood vessels and adrenal glands. Animals have been housed and cared for in accordance with AAALAC standards and Institutional Animal Care and Use Committee guidelines. All studies have approval from the Institutional Animal Care and Use Committee. The total number of animals used has been reduced since investigators that study isolated blood vessels obtain these from a common shared source animal that services the needs of multiple laboratories, thereby providing the maximum yield of tissue using the minimal number of animals.

Animal Instrumentation. Dogs are anesthetized with pentobarbital (30 mg/kg, maintained with supplemental doses of 10 mg/kg). Animals are intubated and mechanically ventilated to maintain normal arterial blood gases. Body temperature is maintained at 36-38°C. Arterial pressure is monitored with an indwelling catheter located in a femoral artery. Central venous pressure (CVP) and cardiac output (some studies) are measured using thermodilution with a pulmonary artery catheter advanced from the femoral or jugular vein. Regional (mesenteric, iliac, renal, or coronary) arterial blood flows are measured using *Transonic* flow probes placed around the vessels of interest after laparotomy or sternotomy. Arterial and central venous pressure, along with regional blood flows, are measured continuously. Arterial blood gases and cardiac output are measured every 10-15 minutes. In some studies, small polyethylene cannulas are placed into the proximal portions of the arteries of interest for local infusion of drugs. After physiological measures are complete, the anesthetized animals are sacrificed using potassium chloride, barbiturate overdose, or exsanguination.

XL-Hgb Transfusion. XL-Hgb from the USAMRDC has been used in two basic transfusion protocols. Hypovolemic partial exchange transfusions are conducted after removal of 30-40 cc/kg of blood or sufficient blood volume to cause an ~30% reduction in mean arterial pressure (MAP). This is followed immediately by resuscitation with 60 cc bolus doses of XL-Hgb sufficient to return MAP to baseline. Sham control experiments will also be conducted using the approaches outlined above, but the volume replacement consists of commercially available albumin or dextran solutions designed to have a viscosity and osmolality similar to that of the XL-Hgb product. Volume expansion has also been used to expand the blood of a normovolemic animal by 10-20% with either XL-Hgb, albumin, or dextran. Total hemoglobin is estimated measuring the hematocrit and the Hgb concentration in spun plasma.

Drugs. The compounds used have been used previously by the investigators, and the appropriate concentrations and doses are selected from their experience and from the literature. Drugs are prepared fresh daily. In studies on isolated tissues where the potency of various drugs are frequently compared, measurements are made on quiescent rings or rings constricted to a similar level of tension [the effective concentration causing 50% (ED_{50}) of the maximal activation of the smooth muscle]. The response to increasing concentration of an agonist is obtained by adding the agonist cumulatively by half-log units to the preparation.

Chemical Determinations:

Catecholamines in the presence of XL-Hgb. Over the past 13 years, the laboratory of Drs. Tyce and Rorie has developed many of the standard catecholamine assays used internationally which yield recoveries of >80% of added authentic standards. It has been impossible to recover catecholamines from fluids containing XL-Hgb using any of these methods. Additionally, a number of catecholamine metabolites appear to be present in the presence of XL-Hgb. These have been assayed using modifications of the standard techniques as described in the original contract. For some of the metabolites, Drs. Rorie and Tyce collaborate with the Mayo Mass Spectrometry Core to identify these compounds.

Cyclic GMP. Basal and stimulated production of cyclic GMP have been analyzed in the large artery rings by RIA. Cyclic GMP is extracted from the tissues by freezing the tissues in liquid nitrogen followed by pulverization and homogenization in trichloroacetic acid. After centrifugation, cyclic GMP is extracted from the supernatant using H^{20} -saturated ether. The DNA of each tissue is assayed fluorometrically and cyclic GMP levels will be expressed as fmol/mg DNA.

NO (Bioassay). Arteries (40 to 45 mm long) prepared to obtain leak-free segments are connected at both ends to stainless steel cannulas in an organ bath maintained at 37°C and filled with aerated (95% O_2 -5% CO_2) control solution. They are then perfused at 1 ml/min using a roller pump (arterial line). A stainless steel tube placed parallel to the vessels is perfused at the same rate (direct line). Rings of canine coronary artery without endothelium are connected to an isometric force transducer. They are superfused with the solution from either the artery segment or the stainless steel cannula, and changes in the tension of the coronary ring are used to detect any biological activity in the outflow from the perfused arteries.

Isolated Blood Vessel Preparations:

Tensions developed in blood vessels. Studies have been conducted in organ baths on isolated canine mesenteric, renal, left circumflex coronary, femoral, and other large arteries. Blood vessels with and without endothelium are prepared using the standard techniques that remove endothelial cells, but preserve the ability of the vascular smooth muscle to contract. The presence or absence of endothelium is confirmed by determining whether or not bradykinin causes relaxation during contractions evoked by an EC_{50} concentration of contractile agonists. All experiments are performed in the presence of indomethacin in order to inhibit activity of cyclooxygenase. Each experimental group consists of at least six preparations from different donors. The role of the endothelium is determined by comparing the responses of arteries with and without endothelium. Whenever possible, experiments are conducted in parallel using tissues derived from the same animal with and without endothelium or with and without a particular antagonist. This will account for the time-dependent changes in responsiveness of the tissues.

NE release from sympathetic nerve endings in vessels. Release of NE and its major neuronal metabolite 3,4-dihydroxyphenylglycol (DOPEG) is measured in isolated canine mesenteric, iliac, and renal

arteries. DOPEG, the deaminated metabolite of NE, is of importance because its concentration in basal superfusate increases together with NE when vesicular retention of NE is impaired. Large arteries are cut into helical strips, mounted in a superfusion apparatus at 37°C, and superfused with Krebs-Ringer at 2 ml/min. Platinum wire electrodes are used for adrenergic stimulation using rectangular waves (10 V; 0.2 ms; 2 Hz) delivered via switching transistor and Grass stimulator. In some experiments, the amount of NE released (rather than the amount which overflows) is studied with desipramine (10^{-6} M), corticosterone (4×10^{-5} M), and yohimbine (10^{-7} M) added to perfusates throughout to inhibit neuronal and extraneuronal uptakes and presynaptic α_2 -adrenoreceptors, respectively. In the presence of these drugs a more nearly true measure of NE release is possible. NE and DOPEG are measured in all superfusates and in tissues after perfusion and stimulation.

Perfusion of the isolated adrenal gland. Adrenal glands are removed from dogs and perfused retrogradely at 1.5 ml/min with Krebs-Ringer containing glucose (11 mM) via the adrenolumbar vein (4) using the technique routinely used by Drs. Rorie and Tyce and previously described in the contract. A basal sample (B1) is first collected over a 10-minute period. A 2-minute adrenal stimulation is then performed with a cholinergic (either muscarinic, nicotinic, or mixed) agonist. Perfusate is collected over the next 2 minutes, and the subsequent 8 minutes is used to measure the evoked release of catecholamines and vasoactive substances (S1). After a 30-minute period, this procedure is repeated. After a further 30-minute rest period and collection of a third basal sample (B3), the perfusing fluid will be switched to (a) XL-Hgb or (b) Krebs-Ringer containing unmodified oxyhemoglobin (both with 11 mM glucose). A further 5-minute basal sample (B3X) will be collected so that the effects of treatments on basal release can be assessed. The same stimulation procedure and sample collection will then be repeated and a 30-minute and a 10-minute post-stimulation sample will be collected. The catecholamines NE, E, and DA; and neuropeptides ME, NPY, PYY, and VIP will be measured in the perfusates. The concentrations of these autocooids will be measured in the adrenal gland after perfusion and also in contralateral non-perfused adrenal glands to determine whether there are depletions in autocooid concentrations after perfusion and stimulation.

PROJECT 1

"Effects of XL-Hgb on Endothelial Regulation of Vascular Tone"

Dr. Katušić

XL-Hgb is a potent vasoconstrictor, and when injected into systemic circulation of experimental animals and humans, it causes an increase in arterial blood pressure (6,8). The mechanisms of hemoglobin-induced vasoconstrictions are not completely understood. Previous studies demonstrated that hemoglobin is an inhibitor of arterial relaxations mediated by relaxing factor(s) released from endothelial cells. This effect has been ascribed to chemical inactivation of endothelium-derived nitric oxide and it may contribute to increased arterial tone observed following infusion of XL-Hgb. The major goal of this project is to characterize the effect of XL-Hgb on endothelium-dependent relaxations and to determine its effect on endothelial L-arginine/nitric oxide pathway.

We performed our experiments on isolated canine femoral, renal, mesenteric, coronary, and basilar arteries. The effects of increasing concentrations of XL-Hgb (10^{-7} , 10^{-6} , and 10^{-5} M) on endothelium-dependent relaxations to acetylcholine and calcium ionophore A23187 were studied. Effects of XL-Hgb were compared with effects of equimolar concentrations of oxyhemoglobin. Initially, the contribution of L-arginine pathway to endothelium-dependent relaxations was determined by studying the effect of nitric oxide synthase inhibitors and L-arginine. In order to more precisely characterize the effect of XL-Hgb on endothelial L-arginine pathway, productions of cyclic GMP and cyclic AMP were measured.

XL-Hgb consistently reduced endothelium-dependent relaxations in response to acetylcholine and A23187. We did not detect any major difference between the effects of XL-Hgb and oxyhemoglobin. In concentration of 10^{-7} M, XL-Hgb was capable of producing almost fifty percent reduction in endothelium-dependent relaxations. This inhibitory effect was associated with a significant reduction in cyclic GMP production in smooth muscle cells. XL-Hgb reduced basal production of cyclic GMP and abolished acetylcholine-induced increase in cyclic GMP. In contrast, XL-Hgb did not affect production of cyclic AMP. These results demonstrated that XL-Hgb impairs endothelium-dependent relaxations in isolated canine arteries. Selective inhibition of cyclic GMP production indicated that the endothelial L-arginine pathway is dysfunctional in the presence of XL-Hgb. It appears that vasoconstrictor and pressor effects of XL-Hgb may be mediated in part by inactivation of endothelial L-arginine pathway. Our studies provided evidence that in large arteries solution of XL-Hgb impairs function of vascular endothelium. These findings may have wider implications for our understanding of the effects of XL-Hgb not only on vascular tone, but on interaction between endothelium, platelets, and white blood cells.

PROJECT 2

"Effects of XL-Hgb Solutions on Catecholamine Release from the Adrenal Medulla and Vascular Sympathetic Nerve Terminals" Drs. Tyce and Rorie

Introduction: Pilot studies showed that substitution of Krebs' Ringer solution with XL-Hgb caused substantial increases in releases of norepinephrine (NE), epinephrine (E), and dopamine (DA) from the isolated perfused dog adrenal gland. This finding was significant in view of the hypertension often associated with the use of XL-Hgb. An overall aim of these studies was to substantiate this finding and to elucidate the mechanisms involved. A further aim was to determine whether XL-Hgb had similar effect on the release of NE from sympathetic nerve endings in blood vessels.

Experiments were done (a) to develop the methodology for measurement of catecholamines in the presence of XL-Hgb, (b) to study the effects of XL-Hgb on releases of catecholamines and the neuropeptides NPY and metEnkephalin (met-Enk) from isolated perfused dog adrenal glands, and (c) to study the effects of XL-Hgb on releases of catecholamines from isolated superfused segments of canine iliac, mesenteric, and renal arteries and portal vein.

Methodology for measurement of catecholamines in hemoglobin solution: The usual methods for measurement of catecholamines from plasma or perfusates (alumina adsorption or cation-exchange chromatography) are not useful for measurement of catecholamines in hemoglobin solutions because the recoveries of added standard are very low (<30%). A new method has been developed using Sephadex G-10 for separation of catecholamines from hemoglobin solutions. This method yields excellent recoveries (75 to 80%). We have also modified our Sep-Pak procedures (described in Anal Biochem 173: 340, 1988) and the modification yields good recoveries.

A number of investigators have employed ultrafiltration (10,000 M.Wt. cutoff and regenerated cellulose membrane) to remove XL-Hgb from solutions prior to routine laboratory tests. We have tested these filtration procedures and shown that XL-Hgb is indeed effectively removed from solutions, but the recovery of added catecholamines was <10% through these procedures.

Releases of catecholamines for adrenal medulla: Initial experiments focused on defining the role of nitric oxide (NO) in modulation of NE, E, and DA releases from the chromaffin cells in the adrenal medulla in isolated perfused dog adrenal glands. These experiments preceded those studying the effects of XL-Hgb since we hypothesized that XL-Hgb would exert its actions via an interaction with NO.

These studies were done to determine the effects of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase without actions at cholinergic receptors, on releases of catecholamines from perfused dog adrenal glands. L-NMMA increased basal releases of NE, E, and DA in a partially Ca²⁺-dependent manner. 3-Morpholinopsydnominine (Sin 1) and to a lesser extent sodium nitroprusside, both sources of NO, decreased effluxes of catecholamines from the adrenal glands. It is concluded that basal releases of catecholamines are inhibited by endogenous and exogenous NO. Inhibition of NO production attenuates this restraint and thus overflow of catecholamine increases.

In addition, increased production of NO appears to inhibit vesicular storage of catecholamines since the production of 3,4-dihydroxyphenylglycol, the metabolite of NE and E produced in the cytoplasm by the action of monoamine oxidase, increases when NO production is increased.

Releases of catecholamines from sympathetic nerve endings in blood vessels: Effects of XL-Hgb:
In isolated superfused segments of iliac, mesenteric, and renal artery and of portal vein, we have never seen increased effluxes of NE in the presence of XL-Hgb. Such an effect would have been in accordance with the hypertension which is associated with the use of this blood substitute. However, we have seen that in all vessels a compound with many of the biochemical properties of dopamine is released during electrical stimulation of the vessels. This release occurs in a frequency-dependent manner. We have worked with Dr. Stephen Naylor of our Mass Spectrometry Facility in attempting to identify this dopamine-like compound. We have also shown that XL-Hgb has a peroxidase-like action on dopamine probably producing dopachrome. Present data suggest that DA is released from the blood vessels during electrical stimulation in the presence of XL-Hgb. This DA is rapidly metabolized by the peroxidase reaction of XL-Hgb. It will be important to determine whether the proposed $DA \rightarrow$ dopachrome reaction occurs before or after DA leaves the junctional cleft, since in most of the blood vessels we have studied we have demonstrated the presence of DA receptors which modulate the release of NE. It would be expected that dopachrome produced within the junctional cleft would have reduced activity at these DA receptors.

PROJECT 3

"Effects of XL-Hgb Solution on Local and Baroreflex Control of Resistance"

Dr. Joyner

Studies to date have addressed issues related to in vivo vascular responses to administration of the vasodilating substances acetylcholine and nitroprusside inter-arterially. Twenty-four acutely instrumented anesthetized dogs have been studied. Flow probes have been placed around the left circumflex coronary artery, the mesenteric artery, and the femoral artery. These vessels have been selectively cannulated and the effects of increasing doses of acetylcholine (ACh, 1-100 mics/min) and nitroprusside (NTP, 1-100 mics/min) have been administered. The animals have also received systemic doses of alpha and beta blocking drugs to blunt any reflexogenic changes in arterial blood pressure in response to the various protocols. The first group of animals (n=6) served as a time control, and successive dose response curves to ACh and NTP were conducted in the vessels of interest. The second group of animals (n=6) underwent an initial series of ACh and NTP dose response curves followed by rapid removal of roughly one-third of their blood volume. This volume was then replaced with albumin and the dose response curves repeated. The third group of animals underwent a similar bleeding protocol, but were resuscitated with an equal volume of cross-linked hemoglobin (~400-500 mls). The fourth group of animals (n=6) underwent dose response curves during control conditions after treatment with the nitric oxide synthase inhibitor L-NMMA, and again after volume loading with cross-linked hemoglobin.

Administration of successive doses of acetylcholine resulted in an increase in blood flow from 2-500%, depending on the blood vessel (coronary \geq femoral which was $>$ mesenteric). Similar dose response relationships were observed with the nitroprusside administration. When no transfusion protocol was performed, these responses were stable with time and repeated dose response curves to both agents were nearly identical. In the second protocol, the blood flow (vasodilation) responses to ACh and nitroprusside were similar to those in group 1. With bleeding, there was a profound drop in arterial pressure from approximately 75-80 mm Hg to 40-50 mm Hg. This fall in pressure was promptly restored by administration of albumin. After albumin administration, dose response curves were again similar, and the reduced viscosity associated with a lower hematocrit appeared to have minimal effect on these relationships. In group 3, the pre-bleeding/transfusion responses were similar to the first two groups. The fall in arterial pressure with blood removal was similar to that observed in group 2. However, when volume resuscitation was performed with XL-Hgb, there was a prompt restoration in arterial blood pressure, and arterial pressure eventually rose to ~120 mm Hg ($p < 0.05$ vs control). This rise in pressure was associated with vasoconstriction in the mesenteric and femoral beds. There was mild vasodilation in the coronary bed, perhaps as a result of the increased blood pressure and subsequent increased metabolic rate of the heart. Dose response curves to acetylcholine and nitroprusside were minimally affected in the mesenteric and femoral arteries. The changes in flow, conductance, or calculated resistance were blunted with ACh administration in the coronary artery. The responses in the coronary artery to nitroprusside were unaffected by XL-Hgb.

In group 4, the control dose response curves to both agents were again similar. Systemic administration of L-NMMA caused a 20-30 mm rise in arterial pressure. This blunted the ACh dose response curve in the coronary artery, but not the femoral and mesenteric arteries. With subsequent XL-Hgb administration, there was an additional increase in arterial pressure.

The following preliminary conclusions can be drawn from our work thus far.

1. Our animal preparation was stable with time, so the effects of any intervention were not due to time.
2. After acute bleeding and volume replacement with albumin, little change in the dose response relationships to ACh or NTP were noted in the coronary, femoral, or mesenteric vascular beds.
3. Cross-linked hemoglobin caused marked systemic and pulmonary hypertension. This was accompanied by generalized vasoconstriction in all vascular beds studied. However, XL-Hgb only caused dramatic effects in the vasodilator responses to ACh and NTP in the coronary arteries.
4. Finally, XL-Hgb can cause hypertensive effects greater than those with L-NMMA on the basis of NO synthase blockade. This suggests, along with the failure of XL-Hgb to blunt NO-mediated vasodilation in response to various drug treatments, that other mechanisms may contribute to the vasoconstriction after XL-Hgb administration. Since these animals were under systemic alpha and beta blockades, it is unlikely that catecholamines could explain these effects (9,10,11). It would appear reasonable to suggest that either XL-Hgb has some sort of other vasoconstricting effect, or that renal mechanisms which can promote hypertension were activated after XL-Hgb administration. We are now attempting to follow up on these issues in isolated blood vessels in further studies on instrumented animals.

PROJECT 4

"Effects of XL-Hgb Solution on Renal Blood Pressure Regulating Mechanisms" Dr. Romero

We have reported before that XL Hb volume expansion performed in six dogs (10% blood volume infusion) produced a decrease in mesenteric (from 210 to 145 ml/min), in renal (from 198 to 131 ml/min), and iliac (from 134 to 73 ml/min) blood flow while mean arterial pressure increased from 114 to 150 mm Hg. In these XL Hb volume expanded animals, urine flow rate, urinary sodium excretion, and glomerular filtration rate remained unchanged despite the increments in arterial pressure. It was then speculated that these hemodynamic and renal function alterations were specifically produced by the scavenging effects of XL Hb, and that to a large extent the same effect should be produced by the administration of nitric oxide inhibitors. A pilot study was conducted in one animal weighing 18 kg where all the previously mentioned parameters were recorded during two periods of 20 min each. After this basal observation, the animals received intravenously the administration of an NO synthesis inhibitor, L-nitro arginine methyl ester (L-NAME), at the rate of 1 $\mu\text{g/kg/min}$; and after 20 min of stabilization, the doses of L-NAME inhibitor were increased up to 50 $\mu\text{g/kg/min}$.

Mean arterial pressure values in the control period were 94 and 96 mm Hg and were increased to 98 and 116 mm Hg respectively during the two doses of L-NAME. Renal blood flow was transiently increased from 125 to 140 mm Hg by the small dose of L-NAME, whereas it came back to control value of 122 mm Hg during administration of 50 $\mu\text{g/kg}$. Glomerular filtration rate was not significantly changed by any of these doses, whereas urine volume was mildly increased from 2.4 to 2.8 ml/min. Coronary and renal blood flow remained unaltered, whereas mesenteric and iliac blood flow experienced a very significant decrease.

These preliminary data revealed that in fact the administration of nitric oxide inhibitors could mimic the effects of XL Hb since it increases mean arterial pressure without increasing natriuresis. An increase in urine volume is likely to reflect specific alterations in the reabsorption of water which has also been observed by Dr. Lahera in rats (Lahera et al, Am J Physiol 261 [Renal Fluid Electrolyte Physiol 30]: F1033-F1037, 1991). We were planning to report at the present time four additional dogs, but this work has been suddenly interrupted by a serious disease of the lead technician, Mary Olsen, who was conducting these experiments. We are now taking appropriate measures to overcome this problem.

We expect to finish this observation because they are clinically and physiologically important. In fact, this is the first time that the exact alterations induced by XL Hb and by the inhibition of nitric oxide in general circulation and renal function are matched. The precise hemodynamic and renal consequences of these two maneuvers are critical to derive conclusions with respect to clinical use of XL Hb.

CONCLUSIONS

Data collected to date in all four components of this contract are consistent with the concept that XL-Hgb scavenges NO and interferes with normal function of this molecule and physiologic regulation. This regulation includes: 1) maintenance of normal basal blood vessel tone; 2) blood vessel responses to vasodilating stimuli; 3) NO control of catecholamine release from adrenal medulla and sympathetic nerves; and 4) NO control of renal function in the face of volume expansion and hypertension. Thus far, the data are completely consistent with the hypothesis that hypertension after XL-Hgb transfusion in animals is caused by disruption of NO function. However, these effects may not be the only causes of hypertension after XL-Hgb administration. Data obtained as part of projects 1 and 3 can be interpreted to suggest that the possibility of substantial NO-mediated vasodilation continued after XL-Hgb administration. In this context, it appears that there may be other vasoconstricting effects of the Hgb itself, or the Hgb molecules may interfere with other vasodilating pathways or activate vasoconstricting pathways. *Studies conducted in the second half of this contract should identify the extent to which these mechanisms contribute to the hypertensive effects of XL-Hgb administration.*

Evidence from project 2 also continues to indicate that XL-Hgb has marked effects on the metabolism of catecholamines in the adrenal medulla sympathetic nerves. Such effect might further contribute to the hypertensive effects of XL-Hgb administration. Additionally, the general difficulty of assaying for biological compounds in the presence of XL-Hgb may present a major challenge to such products wherever routinely used in injured humans. Prior to use of these products in humans it will be necessary to determine what impact they have on normal clinical chemistry values that frequently guide the therapy of severely injured patients in a critical care unit.

Data from project 4 indicates that XL-Hgb disrupts normal renal blood pressure and blood volume regulating mechanisms. Such disruption could serve to maintain the hypertensive state with time after XL-Hgb administration.

REFERENCES

1. Alayash AI, Fratanoni JC, Bonaventura C, et al. Nitric oxide binding to human ferrihemoglobins cross-linked between either α or β subunits. *Arch Biochem Biophys* 1993;303:332-8.
2. Amberson WR, Mulder AG, Steggerda FR, et al. Mammalian life without red blood corpuscles. *Science* 1933;78:106-7.
3. Amberson WR, Flexner J, Steggerda FB, et al. On the use of Ringer-Locke solutions containing hemoglobin as a substitute for normal blood in mammals. *Journal of Cellular and Comparative Physiology* 1934;5:359-82.
4. Chatterjee R, Welty EV, Walder RY, et al. Isolation and characterization of a new hemoglobin derivative cross-linked between the α chains (lysine 99 α_1 \rightarrow lysine 99 α_2). *J Biol Chem* 1986;261:9929-37.
5. Fratanoni JC. Points to consider in the safety evaluation of hemoglobin-based oxygen carriers. *Transfusion* 1991;31:369-71.
6. Hess JR, Wade CE, Winslow RM. Filtration-assisted exchange transfusion using $\alpha\alpha$ Hb, an erythrocyte substitute: *J Appl Physiol* 1991;70:1639-44.
7. Hess JR, Fadare SO, Tolentino LSL, et al. The intravascular persistence of crosslinked human hemoglobin. *Prog Clin Biol Res* 1989;319:351-7.
8. Hess JR, MacDonald VW, Brinkley WW. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. *J Appl Physiol* 1993;74:1769-78.
9. Katušić ZS, Vanhoutte PM. Endothelium-dependent contractions to N^G-monomethyl-L-arginine in canine basilar artery. In: Rubanyi GM, Vanhoutte PM, eds. *Endothelium-Derived Relaxing Factors*. Basel: Karger, 1989:95-8.
10. Macdonald RL, Weir BKA. A review of hemoglobin and the pathogenesis of cerebral vasospasm. *Stroke* 1991;22:971-82.
11. Martin W, Villani GM, Jothianandan D, Furchgott RF. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J Pharmacol Exp Ther* 1985;232:708-16.
12. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-42.
13. Snyder SR, Welty EV, Walder RY, et al. GbXL99 α : a hemoglobin derivative that is cross-linked between the alpha subunits is useful as a blood substitute. *Proc Natl Acad Sci USA* 1987;84:7280-4.
14. Webster NR. Battlefield transfusions. *JAMA* 1994;271:319.

15. Winslow RM. Blood substitutes - minireview. In: Brewer GJ, ed. Proceedings of the Seventh Ann Arbor Conference, Ann Arbor, MI: Prog Clin Biol Res 1989;319:305-23.
16. Winslow RM. Red cell substitutes: current status, 1992. In: Nance SJ, ed. Blood Safety: Current Challenges. Bethesda, MD: American Association of Blood Banks, 1992:151-67.